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Photo-triggered destabilization of nanoscopic vehicles by dihydroindolizine for enhanced anticancer drug delivery in cervical carcinoma

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ABSTRACT

The efficacy and toxicity of drugs depend not only on their potency but also on their ability to reach the target sites in preference to non-target sites. In this regards destabilization of delivery vehicles induced by light can be an effective strategy for enhancing drug delivery with spatial and temporal control. Herein we demonstrate that the photoinduced isomerization from closed (hydrophobic) to open isomeric form (hydrophilic) of a novel DHI encapsulated in liposome leads to potential light-controlled drug delivery vehicles. We have used steady state and picosecond resolved dynamics of a drug 8-anilino-1-naphthalenesulfonic acid ammonium salt (ANS) incorporated in liposome to monitor the efficacy of destabilization of liposome in absence and presence UVA irradiation. Steady state and picosecond resolved polarization gated spectroscopy including the well-known strategy of solvation dynamics and Förster resonance energy transfer; reveal the possible mechanism out of various phenomena involved in destabilization of liposome. We have also investigated the therapeutic efficacy of doxorubicin (DOX) delivery from liposome to cervical cancer cell line HeLa. The FACS, confocal fluorescence microscopic and MTT assay studies reveal an enhanced cellular uptake of DOX leading to significant reduction in cell viability (~40%) of HeLa followed by photoresponsive destabilization of liposome. Our studies successfully demonstrate that these DHI encapsulated liposomes have potential application as a smart photosensitive drug delivery system.

1. Introduction

Potential therapeutic (cargo drug) encapsulation coupled with stimuli responsiveness of drug delivery systems (DDS) have gained tremendous attention in recent years [1,2]. It not only eludes the possibilities of premature drug release but also improves the therapeutic efficacy by the means of enhanced spatio-temporal accumulation of the therapeutic payload [3,4]. This further lowers the cytotoxic effect of the therapeutic drug other than the tumor sites and also favors overcoming the drug resistance [5,6]. These classes of DDS are mostly composed of three ingredients (i) a tumor targeting therapeutic carrier (ii) an external stimuli sensitive agents that can destabilize therapeutic carrier for efficient drug delivery (iii) a drug capable for tracking the spatial distribution, localization, and depletion from the DDS. Owing to the encapsulation characteristics and biocompatibility, liposomes recommend them as efficient carriers for therapeutic agents [7]. Out of the various published methods for triggering the release of drug molecules from liposome, photoinduced destabilization is a particularly attractive method for providing fast reaction rates [8]. Photo induction or light triggering generally offers the great benefit of not affecting physiological parameters such as temperature, pH and ionic strength, a fundamental requisite for biomedical applications [9,10]. Liposome can be made photosensitive by using photochromic agents that isomerize [11–14], polymerize [15–17], fragment [18] or induce oxidative reactions [19,20] upon irradiation [21,22].

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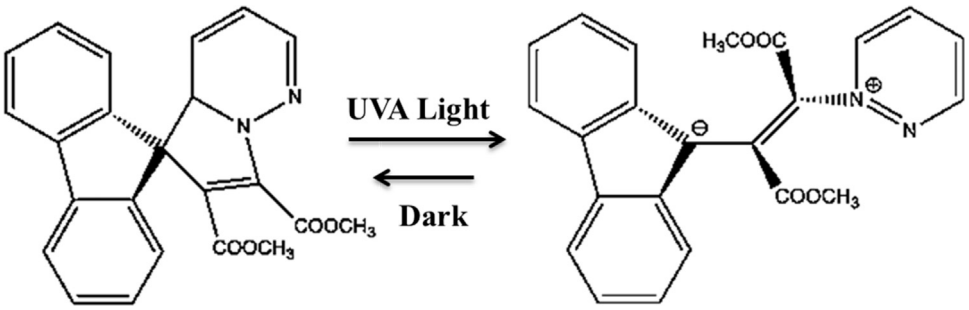


Scheme 1. Structures of the closed and open isomers of DHI.

Many studies have reported changes in the permeability of vesicles toward ions and low molecular weight water-soluble compounds upon irradiation [15,23]. In contrast, biopolymers do not easily cross the membrane owing to the combined effects of conformation [24] and electric charge [25] and a drastic change in liposome morphology is required. Riske et al. reported morphological changes (bi-layer destabilization) in vesicles of mixtures of phosphatidylcholine and amphiphilic porphyrin which works as a photosensitizer [20]. Although the morphological changes (bi-layer destabilization) observed are remarkable but their system has a high molar ratio of photoresponsive amphiphile to membrane lipid (>40 mol%), which is in comparable to the composition of biomembranes. In this regard, investigations have been conducted on liposomes containing photoresponsive compounds such as azobenzene, stilbene, spiropyran [14,23,26], and photo-polymerizable lipids [15,16], wherein the lipid membrane is destabilized by light. Photosensitive proton sources such as 3,3-dicarboxydiphenyliodonium salts allow for efficient destabilization of the phosphatidylcholine bilayer by adsorption of the hydrophobic polyelectrolyte, but result in vesicle-to-micelle transition, and consequently, corroborate complete solubilization of the membrane [27]. Since solubilization of the vesicles results in cytotoxicity, cellular uptake of drugs in the targeted domain without any solubilization is desired for ideal drug delivery systems. Therefore, it is necessary to ensure photo-triggered destabilization of phosphatidylcholine membrane without disruption of the liposome structure.

Herein, we have used a synthesized photochromic dye dihydroindolizine (DHI) to investigate its efficacy in photoresponsive destabilization of phosphatidylcholine liposome, which is used as potential drug delivery vehicles. Photochromic DHI dye has received much attention owing to its remarkable photo-fatigue resistance and broad range of absorption. Specifically, DHI can undergo a reversible transformation from light yellow colored isomer to red-colored betaine isomer upon UVA irradiation with an increase in the polarity associated with the structural conversion from neutral (closed form) to charge-separated zwitterions (open form) (Scheme 1) [28,29]. This structural conversion of DHI from closed to open isomer can fluctuate or defect the liposomal membrane by mechanical stress and leads photoresponsive destabilization to liposome. Besides destabilization, photochromic agents can affect the liposome by various processes including aggregation [30,31], fusion [23,32,33] and total membrane disruption [34]. Hence, we have monitored the entire possible phenomena

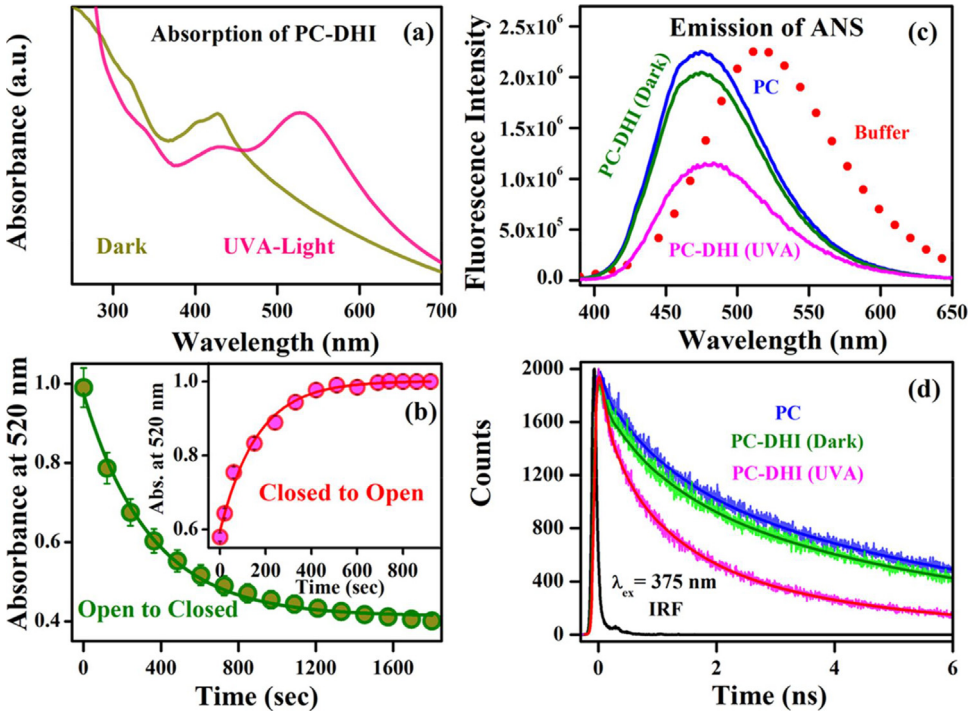


Fig. 1. (a) Absorption spectra of DHI in liposome: closed and open isomers. (b) Kinetics of the open to closed transition of DHI in liposome (SD = ±0.008, n = 3). Inset shows the corresponding closed to open conversion rate (SD = ±0.003, n = 3). (c) Steady state emission spectra of ANS in buffer, ANS bound to PC and PC-DHI in presence and absence of UVA light. (d) Time-resolved transients of ANS, ANS bound to PC and ANS bound to PC-DHI in the presence and absence of UVA light.

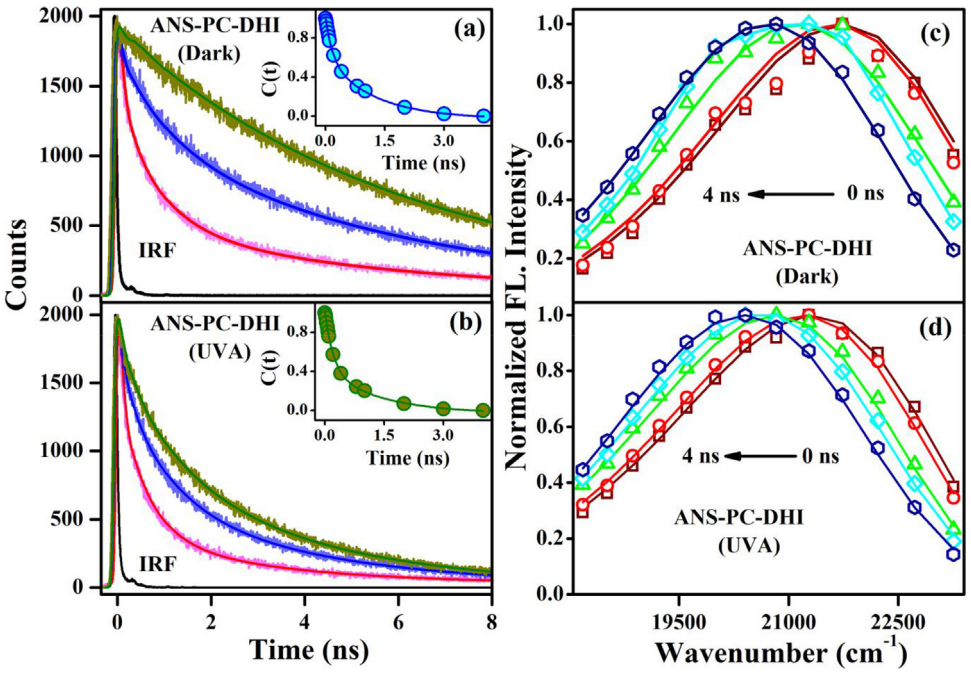


Fig. 2. (a) Picosecond-resolved transient of ANS at three different wavelengths in (a) PC-DHI in dark condition and (b) PC-DHI in presence of UVA light. (c, d) Time-resolved emission spectra (TRES) of corresponding systems are shown. Insets depict the corresponding solvation correlation decay profile of ANS.

by using time resolved fluorescence spectroscopy by labelling the liposome with fluorescent probe 8-anilino-1-naphthalenesulfonic acid ammonium salt (ANS). The picosecond resolved fluorescence transients and polarization gated spectroscopy studies of ANS attached to hydrophobic core of liposome confirm higher permeability of liposome upon closed to open isomerization of DHI. The solvatochromic property of ANS has been used to study the dynamics of solvation and also to eliminate the possible existence of membrane disruption (like membrane solubilisation and vesicles to micelles conversion) in the studied systems upon light irradiation. Förster resonance energy transfer (FRET) techniques between the FRET pair ANS (incorporated to one set of liposome) and doxorubicin (encapsulated to other liposome system) have successfully manifested the possibility of liposome fusion by the course of photo triggered bilayer destabilization. Based on these photoresponsive microstructural changes of liposome, the in vitro drug release profile of cargo drug (DOX) has been studied through dialysis method. We have also investigated the therapeutic efficacy of DOX delivery from liposome to cervical cancer cell line HeLa. Our FACS, confocal fluorescence microscopic and MTT assay based studies reveal an enhanced cellular uptake of DOX leading to significant reduction in cell viability of HeLa due to photoresponsive destabilization of liposome. Hence, we successfully established the DHI encapsulated liposome as a promising and model drug delivery vehicle.

2. Experimental section

2.1. Chemicals

L-α-Phosphatidylcholin from soybean (PC), 8-anilino-1-naphthalenesulfonic acid ammonium salt (ANS) and doxorubicin (DOX) were obtained from Sigma-Aldrich (Saint Louis, USA). All reagents were used without further purification. Ethanol (Merck, India) and water (Milli-Q, USA) were used as solvents. The solutions were prepared in 100 mM phosphate buffer (pH 7.4) using water from Millipore.

2.2. Synthesis of photochromic DHI

The photochromic DHI was synthesized [35] by the electrophilic addition of electron-deficient spirocyclopropenes through the nitrogen atom of the N-heterocyclic pyridazines in dry ether in the absence of light under nitrogen atmosphere for 24 h. The final photochromic DHI was obtained as pale yellow crystals after recrystallization from the proper solvent. Pure products were obtained after purification by column chromatography on silica gel using dichloromethane as eluent [36].

2.3. Synthesis of liposomes

The liposomes were prepared by using the standard ethanol injection methods [37]. Typically, the phospholipids were dissolved in the ethanol solution and then injected rapidly in PBS buffer (pH 7.4) followed by vigorous stirring for half an hour. Finally, the ethanol and a part of water were removed by rotary evaporation under reduced pressure. As in present studies our motive is to investigate the mode of liposome destabilization, which are found to be independent on manner of liposome formation, we have used relatively easier ethanol injection technique rather extrusion method for liposome preparation [38].

2.4. Preparation of liposome-DHI solution

Liposome-DHI solutions were prepared by adding a requisite amount of DHI (1.5 mM) to the liposome solution (20 mM) with stirring for 6 h. To ensure complete complexation of DHI with the liposome free DHI was removed by centrifuging the liposome encapsulated DHI at 5000 rpm for 2 min. For destabilization studies, the probes (ANS; 1 mM) were added to the liposome-DHI solution and stirred for 6 h. A UVA light source (LED) of 400–410 nm and ~50 mW/cm^2 power was used to isomerize the DHI solution. To compare photo-isomerization reactions, all the external parameters such as-lamp power, UV irradiation time, temperature and solvent are kept same.

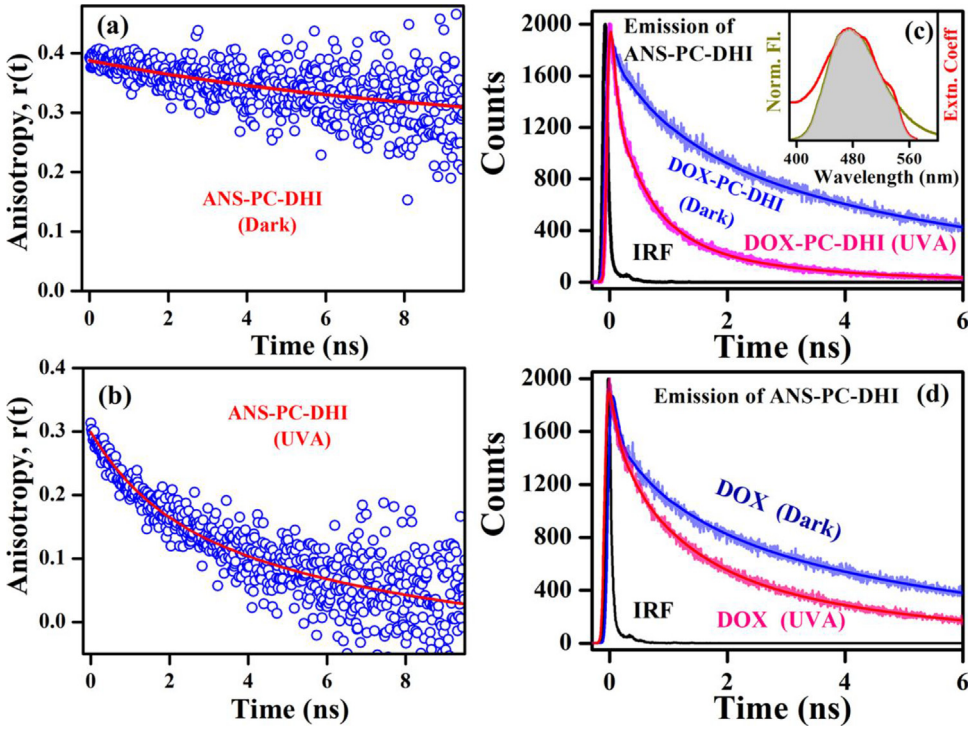


Fig. 3. Time-resolved anisotropy of ANS bound to PC-DHI in presence of (a) dark and (b) UVA light. Picosecond-resolved transients of the donor-acceptor in the absence and presence of UVA light, (c) donor is (ANS-PC-DHI) and acceptor is (DOX-PC-DHI) and (d) donor is (ANS-PC-DHI) and acceptor is free DOX. Insets depict the corresponding spectral overlap between donor (ANS-PC-DHI complex) emission and acceptor (DOX-PC-DHI) absorbance.

2.5. DOX load and release

The DOX solution was prepared in PBS buffer, separately. Then the DOX was dropwise added to the liposome-DHI solution and incubated (70 °C) followed by vigorous stirring for 6 h. Free DOX was removed by centrifuging the liposome encapsulated DOX at 14,000 rpm for 50 min. The absorbance of encapsulated DOX ($A_{encapsulated}$) was obtained by using UV–vis spectrophotometer at 480 nm after destructing liposomes by Triton X-100. The encapsulated efficiency EE was found to be 68.5% was calculated by following equation:

$$EE\% = (A_{encapsulated}/A_{total}) * 100$$

Where A_{total} was the absorbance of DOX added in solution originally.

The drug release behavior was measure by monitoring the released from dialysis tube. For this 1 mL DOX-loaded liposomes-DHI with and without UVA-irradiation for 20 min were transferred to the dialysis tube (MWCO 14 kDa), and then submerged into 250 mL PBS buffer (pH 7.4) solution under stirring condition at 37 °C. The DOX release from the liposomes-DHI was measured by Jobin Yvon Fluorolog fluorimeter. The release efficiency RE of liposomes was calculated using following equation:

$$RE\% = (A_t/A_{encapsulated}) * 100$$

Where A_t was the emission at 480 nm at time t.

2.6. Characterization techniques

The steady state absorption and emission spectra were measured with Shimadzu UV-2600 spectrophotometer and Jobin Yvon fluorolog fluorimeter, respectively. All the picosecond resolved fluorescence transients were measured by using commercially available time-correlated single-photon counting (TCSPC) setup with MCP-PMT from Edinburgh instrument, U.K. (instrument response function (IRF) of ~75 ps) using a 375 nm excitation laser source. Details of the time resolved fluorescence setup have been discussed in our previous reports [39,40]. Time-resolved emission spectra (TRES) and time-resolved area normalized emission spectra (TRANES) were constructed following the methods described earlier[26,27] to determine the time dependent fluorescence Stokes shifts. In brief, the normalized spectral shift correlation function or the solvent correlation function, C(t), is defined as, $C(t) = \frac{\nu(t)-\nu(\infty)}{\nu(0)-\nu(\infty)}$, where $\nu(0)$, $\nu(t)$, and $\nu(\infty)$ are the emission peak maxima (in cm^{-1}) at time 0, t, and ∞ respectively. For the fluorescence anisotropy measurements, the emission polarizer was adjusted to be parallel and perpendicular to that of the excitation and the corresponding fluorescence transients are collected as I_{para} and I_{per}, respectively. The time-resolved anisotropy is defined as, $r(t) = \frac{(I_{para}-G*I_{per})}{(I_{para}+2*G*I_{per})}$. The magnitude of G, the grating factor of the emission monochromator of the TCSPC system, was found using a long tail matching technique. FRET distance between donor – acceptor (r) was calculated from the equation $r^6 = [R_0^6(1-E)]/E$, where E is the energy transfer efficiency between donor and acceptor and following the procedure published elsewhere [41]. The size distribution and hydrodynamic diameter (d_H) of the liposomes-DHI in absence and presence of UVA irradiation were measured from DLS experiments, performed on a Nano S Malvern instrument (4 mW, He–Ne laser, λ = 632.8 nm). Details of the experimental techniques are described in our previous papers [42]. The morphological change in the liposomes-DHI was confirmed by scanning electron microscopy. For this 200 μL of the respective liposome was kept over silicon wafer for 24 h at 37 °C. The silicon wafer were coated with gold and scanned in a field emission scanning electron microscope (Quanta FEG 250: source of electrons, FEG source; operational accelerating voltage, 200 V to 30 kV; resolution, 30 kV under low vacuum conditions: 3.0 nm; detectors, large field secondary electron detector for low vacuum operation).

2.7. Cytotoxicity assay

MTT assay was performed to observe the cytotoxic effect of DOX loaded liposomes and also to assess the photoinduced drug delivery. HeLa cells and HaCaT cell were grown in Dulbecco's modified eagle's medium (DMEM; HiMedia) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1.0% penicillin/streptomycin (HiMedia) and cultured at 37 °C, 5.0% CO_2, and 95% humidity. 1.0×10^4 cells were seeded in each well of a 96 well plate and cultured in 10% FBS-supplemented DMEM for the photoresponsive treatment. Cells were incubated with PC-DHI, PC-DOX, and PC-DHI-DOX (individual concentration of PC and DOX in the complex were 2 mM and 2 μM, respectively) for 1 h and were exposed to UVA light source for 15 min [43]. After 24 h of incubation, the MTT assay was performed using a MTT assay kit (CCK003, Himedia) as per the manufacture's instruction. For in vitro studies, MTT assay was done in similar way by incubating the cell with drug obtained from dialysis of PC-DOX, and PC-DHI-DOX under dark and UVA irradiation, respectively.

2.8. Flow cytometry

5×10^5 cells were seeded in each well of a 6 well plate and cultured in 10% FBS-supplemented DMEM for 12 h. After that the cells were treated with PC-DOX liposome and PC-DHI-DOX liposome both in absence and presence of UVA light. Post- treatment, cells were incubated for 4 h. Then, the doxorubicin uptake by the cells was checked by flow cytometry (BD accuri C6) and data was analyzed using BD accuri C6 software.

2.9. Fluorescence microscopy studies

Micrographs of HeLa cells were taken using Leica TCS SP8 confocal fluorescence microscope by staining cell using Hoechst.

3. Results and discussion

3.1. Photoisomerization of DHI

To elucidate the consequence of photo-sensitive isomerization of DHI to liposome bilayer, primarily the interaction of DHI with liposome (PC) was studied. Fig. 1(a) represents the optical absorption spectrum of the neutral form of DHI in liposome bilayer having peak maxima at 410 nm. A redshift in the absorption spectra of DHI relative to that in acetonitrile solution having absorption maxima at 390 nm (Fig. S1) confirms the ground-state complexation of the photochromic dye with the liposome [44]. The reversible transformation of DHI from closed to open configurations in the liposome was evident from the decrease of the absorption peak at 410 nm and the concurrent increase of the strong peak at 520 nm. These absorption bands can be assigned to the locally excited π–π^* transition that occurs in the butadienyl-vinyl-amine chromophores. To evaluate the photochromic behavior of DHI in liposome, photo isomerization reaction kinetics was monitored by measuring the change in absorbance at 520 nm. While UVA triggered closed to open transition of DHI corroborates increased in absorbance at 520 nm (shown in inset of Fig. 1(b)), whereas thermal relaxation leading open to closed transition of DHI was confirmed by decrease in absorbance at 520 nm (shown in Fig. 1(b)). The values of rate constant (t) for closed to open and open to closed isomerization of DHI were found to be 150 s and 340 s, respectively. The significant increase of t for DHI in liposome with respect to acetonitrile solution was thought to be resulted from hindered and slowed isomerization process due to the incorporation of DHI into the liposomes bilayer.

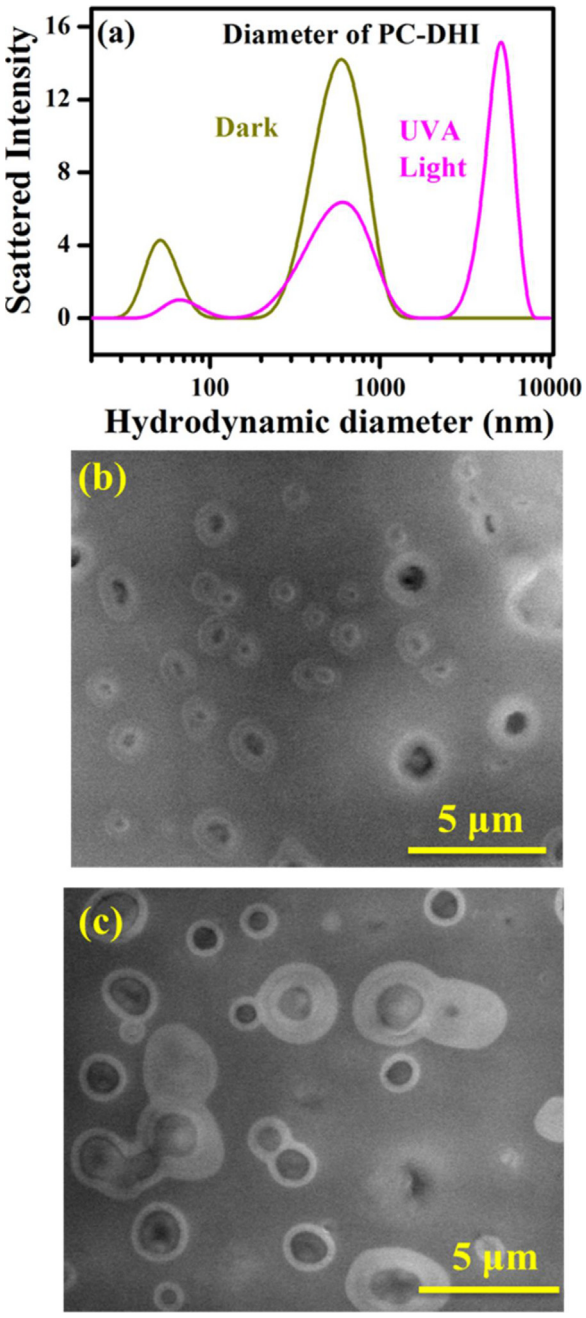


Fig. 4. (a) Hydrodynamic diameter of PC-DHI in absence and presence of UVA light. SEM images of PC-DHI in (b) dark condition and (c) upon UVA-irradiation.

3.2. Process involved in destabilization and morphological studies of liposome

After subsequent validation of the DHI incorporation to liposome, consequence of different isomerization of DHI on liposome stability is monitored by using fluorescent probe 8-anilino-1-naphthalenesulfonic acid ammonium salt (ANS) which is commonly used to monitor the structural changes of proteins and membranes [45]. Fig. 1(c) represents the steady-state fluorescence spectra of ANS in liposome (~480 nm) which is found to be markedly blue shifted compared to buffer (~520 nm) indicating that the local polarity around ANS is lower than that of bulk water. Furthermore, the similarity in the position of the emission peak maximum of ANS in the liposome to that in CHT protein (~480 nm) is consistent with the fact that the dye resides in the hydropho-

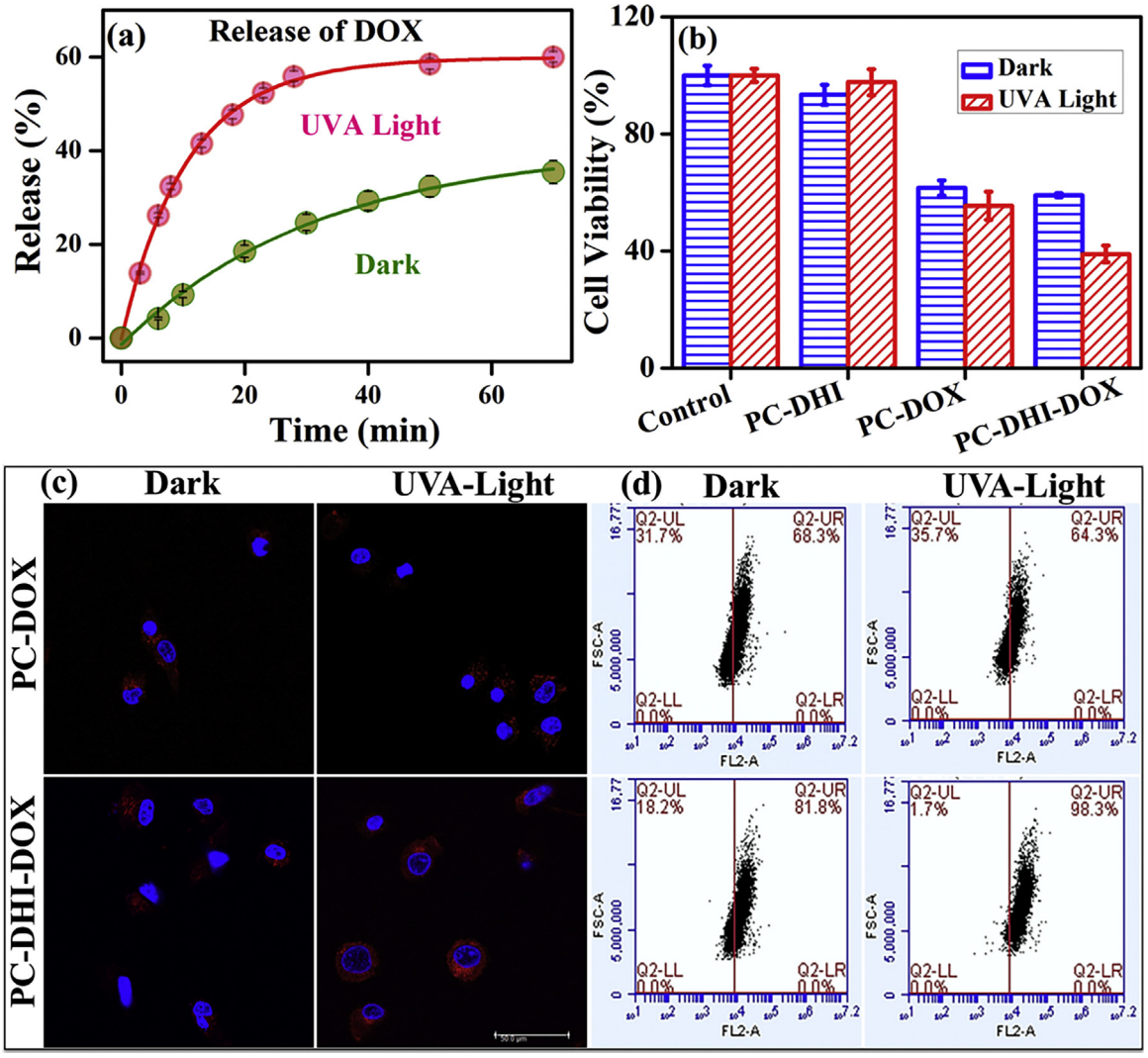


Fig. 5. (a) Release profile of DOX from PC-DHI in absence (SD = ±0.4, n = 3) and presence of UVA light (SD = ± 0.9, n = 3). (b) Cytotoxicity assay in HeLa cells with PC-DHI, PC-DOX, and PC-DHI-DOX with MTT as an indicator dye in the presence and absence of UV light. (c) Confocal microscopy images of HeLa cells treated with PC-DOX liposome and PC-DHI-DOX liposome both in absence and presence of UVA light (d) Flow cytometry of HeLa cells treated with PC-DOX liposome and PC-DHI-DOX liposome both in absence and presence of UVA light.

bic bilayers of the liposome [46]. The fluorescence intensity as well as emission peak maximum of ANS-PC is observed to be similar to ANS-PC-DHI complex under dark condition, revealing the fact that ANS molecules are not detached from PC upon encapsulation of DHI to same hydrophobic bilayer of the liposome. However, the fluorescence intensity of ANS-PC-DHI is found to be decreased along with a small red-shift (~5 nm) in emission maximum upon UVA irradiation. This suggests that ANS is now experiencing higher polarity upon closed to open transition of DHI. The higher polarity around ANS due to UVA irradiation on DHI could occurs either due to displacement of some ANS toward bulk solution or due to increase in permeability of liposome because of its destabilization. Fig. 1(d) shows picosecond-resolved transients of ANS bound PC which is found to be multi-exponential in nature with average decay time constant of 3.0 ns. The fluorescence transient of the ANS-PC-DHI complex is found to be faster upon UVA irradiation (average decay time of 1.3 ns) than that of the dark condition (average decay time of 2.8 ns). The faster fluorescence decay along with the reduced quantum yield of ANS-PC-DHI upon UVA irradiation also indicates the enhancement of non-radiative relaxation due to increase in mobility of the solvating species. The enhancement of polarity around ANS could occur due to photoresponsive destabilization of liposome. As DHI is hydrophobic under dark conditions, it used to encapsulate in the lipid bilayer of liposome. Once irradiated by UVA light, DHI isomerize from closed form to open form. Since the size and polarity of open form of DHI is different (higher) hence the orientation of open form will be different from that of closed form of DHI. Thus, under UVA irradiation, the liposomal membrane would be disturbed by the reorientation of open isomer and leads to increase in membrane permeability by mixing the inner components of the liposomes with outer components in aqueous solution.

The ability to induce destabilization by light suggests many interesting primary processes involved in changing the liposome reconstruction. Beside bilayer destabilization, photoresponsive agent have various effects on liposomes, including fusion [32,33,47], rearrangement of bilayer [30,31] and total membrane perturbation (i.e. transformation into micelles or solubilization of the lipid membrane) [34,48]. However the eminent role of water during the course of perturbation/rearrangement cannot be neglected as it plays a major role in assembly of macromolecular structure and dynamics. Hence to understand the dynamic nature of water molecules at the liposome bilayer during photoresponsive destabilization, time resolved stokes shift (TRSS)

Table 1
Fluorescence lifetimes of ANS in different systems.

System/Wavelength (nm)	$\tau_1 \pm$SD ns [%]	$\tau_2 \pm$SD ns [%]	$\tau_3 \pm$SD ns [%]	τ_{avg} ns	χ^2
PC/480 nm	0.12 ± 0.01(33)	1.04 ± 0.05 (22)	6.22 ± 0.21 (45)	3.07	1.03
PC-DHI/430 nm	0.13 ± 0.01(58)	0.92 ± 0.04 (27)	5.71 ± 0.28 (15)	1.17	1.04
PC-DHI/480 nm	0.12 ± 0.01(35)	1.08 ± 0.05 (23)	6.00 ± 0.30 (42)	2.82	1.05
PC-DHI/550 nm	0.17 ± 0.02 (−5)	0.16 ± 0.01 (29)	6.17 ± 0.31 (76)	4.72	1.05
PC-DHI-UVA/430 nm	0.12 ± 0.01(68)	0.84 ± 0.04 (25)	4.38 ± 0.21 (7)	0.61	1.08
PC-DHI-UVA/480 nm	0.15 ± 0.01(46)	1.04 ± 0.05 (31)	3.99 ± 0.19 (23)	1.31	1.04
PC-DHI-UVA/550 nm	0.18 ± 0.01(38)	1.26 ± 0.06 (28)	3.77 ± 0.18 (34)	1.70	1.03

For various systems, the wavelengths (nm) and standard deviation (SD) of time-resolved decay measurements are shown. The amplitudes corresponding to the relevant decay components are shown within the parentheses.

could be useful techniques to estimate the environmental relaxation of biomolecules over a broad time scale. Fig. 2(a) shows the wavelength-dependent emission transients of ANS in PC-DHI complex under dark condition at three characteristic wavelengths. The time resolved fluorescence at the blue and the red end is characterized by decay and a rise, respectively, indicating the picture of solvation dynamics, a phenomenon which represents the rate at which the solvent dipoles/charged species are rearranged, surrounding an instantaneously created dipole. Upon UVA irradiated closed to open transition of DHI, the transients of ANS in PC still show wavelength dependency, however, with a decrease in the time constants as shown in Fig. 2(b) (Table 1).

Fig. 2(c) and (d) shows the constructed time-resolved emission spectra (TRES) of ANS-PC-DHI with a spectral shift of 1012 cm^{-1} and 959 cm^{-1} in dark and UVA irradiated condition, respectively, in a 4 ns time window. ANS bound to PC-DHI in dark (inset of Fig. 2(a)) exhibits bimodal solvation dynamics with time components of 0.17 ns (40%) and 1.24 ns (60%), respectively. which are consistent with our previous studies on the solvation dynamics of liposome [49]. Upon UVA irradiation, both the two time components becomes faster, indicating increased polarity in the bilayers however presence of longer component represents ANS is still attached to the liposome systems. As both these components are slower than the sub picosecond solvation time scale reported for the bulk water [50] hence we can conclude that UVA irradiated isomerization of DHI could destabilized bilayer without any membrane disruption. To further investigate possible heterogeneity in the positional distribution of ANS attached to liposome we follow time-resolved area normalized emission spectra (TRANES shown in Fig. S2), which is a well-established technique and is a modified version of TRES [51–53]. The unique feature of this method is that existence of an isoemissive point in the spectra indicates the presence of two emitting species in the system (i.e., heterogeneity in the residence of the fluorophore). In the present study, we do not find any isoemissive point when we construct TRANES (Fig. S3) in presence of UVA. This observation confirms the absence of membrane disruption i.e. liposome to micelle transition upon UVA irradiation.

To ascertain the geometrical restriction of the probe in the bilayer region, we measure the time-resolved rotational anisotropy of the ANS in dark and UVA irradiated PC-DHI complex (Table 2). The time-resolved anisotropy decay of ANS-PC-DHI, shown in Fig. 3(a), revealed a rotational time constant of 8.09 ns attributed to the overall global tumbling motion of the liposome. With UVA irradiation the r(t) decay of the ANS-PC-DHI complex (Fig. 3(b)) exhibited one faster component of 0.71 ns due to the internal rotation of the fluorophore relative to the liposome (wobbling of the probe), indicating a progressive release of restriction on the probe might be due to increase in the mobility of solvating species. However presence of a slow component of the rotational time constant indicates ANS is still bound to the surface of the liposome. This change is also manifested in the observed faster solvation dynamics of ANS bound to PC at UVA irradiated closed to open transition of DHI. Thus, the observed correlation between faster solvation dynamics and flexible internal motion of liposome corroborates that UVA irradiated isomerisation of DHI destabilizes the local environment of the bilayers of liposome without membrane disruption. In the latter case the dynamics of solvation is expected to be much faster (bulk water type) than the observed change of average solvation time constant approximately from 810 ps to 580 ps upon UVA exposure.

After revealing that the irradiation of DHI induces liposome destabilization in the absence of total solubilization of the lipid membrane, we examined the possibility of other two processes i.e. rearrangement of bilayer and fusion between liposomes, induced by DHI. To understand the fusion phenomenon, Förster resonance energy transfer (FRET) techniques was used which offers a unique opportunity to measure an efficiency of energy transfer when donor and acceptor comes in proximity of each other. In order to monitor the fusion process, ANS was encapsulated in a group of liposome and doxorubicin (DOX) was encapsulated in another were both the two type of liposome was having DHI. Inset of Fig. 3(c) shows a spectral overlap between ANS-PC-DHI emission and the absorption of the DOX-PC-DHI ($J(\lambda) = 4.4 \times 10^{14}$) suggests a possibility of FRET from ANS to DOX. The average lifetime of ANS in presence of mixer of two liposome decreased from 2.40 ns in dark condition to 0.47 ns upon UVA irradiated (Table 3). The energy-transfer efficiency was calculated to be 80% (see the Experimental Section); as a consequence, the ANS-DOX distance is found to be 2.6 nm. We confirmed in a separate experiment that simple dilution of DOX in the medium containing ANS-PC-DHI does not cause the quenching of ANS fluorescence outside the liposome upon UVA irradiation (Fig. 3(d)). It has to be noted that the marginally faster time constant of ANS-PC-DHI in presence of free DOX upon UVA irradiation is the signature of fast relaxation of ANS due to destabilization of bilayer. Therefore, the quenching of ANS fluorescence may take place when the ANS-encapsulating liposomes are fused together with the DOX-encapsulating liposome in presences of UVA light. The finding also indicates that UVA irradiated DHI sensitized liposome could not induce the rearrangement leading to inclusion of lipids from external sources. The rearrangement would not cause quenching of ANS emission.

The effect of photoresponsive membrane destabilization on the change in liposome morphology was also monitored by investigating the size of liposomes before and after UVA-irradiation. Fig. 4(a) shows the DLS spectra of liposome which was found to be polydispersed with ~40–500 nm of size distribution. Due to UVA-irradiation, these peaks had divided into three peaks at 40 nm, 500 nm and 3000 nm. The significant increase in size also indicates not only the expansion but also the possibilities of fusion of liposomes. The size distribution of liposomes verified the vesicle structures existed in both conditions. Moreover, in order to investigate the liposome fusion at lower concentration, we have performed DLS experiment with lower PC concentration values (data not shown). It is observed that the DHI-sensitized liposomes of ~50 μm PC concentration, undergo fusion upon UVA exposure with relatively lower degree of fusion due to dilution leading to

Table 2
Solvation correlation time constants and rotational time constants of ANS in different systems.

System	Solvation time constants		Rotational time constants		χ^2
	$\tau_1 \pm$ SD ns [%]	$\tau_2 \pm$ SD ns [%]	$\tau_1 \pm$ SD ns [%]	$\tau_2 \pm$ SD ns [%]	
PC-DHI	0.17 ± 0.01(40)	1.24 ± 0.06(60)	–	8.09 ± 0.24 (100)	0.96
PC-DHI-UVA	0.15 ± 0.01(43)	0.91 ± 0.04(57)	0.71 ± 0.0 3(17)	4.66 ± 0.09 (83)	0.88

For various systems, the standard deviation (SD) of time-resolved decay measurements is shown. The amplitudes corresponding to the relevant solvation and rotational components are shown within the parentheses.

Table 3
Fluorescence lifetimes of ANS-PC-DHI in different systems.

System	$\tau_1 \pm$ SD ns [%]	$\tau_2 \pm$ SD ns [%]	$\tau_3 \pm$ SD ns [%]	τ_{avg} ns	χ^2
DOX-PC-DHI	0.11 ± 0.01(47)	1.08 ± 0.05(19)	6.04 ± 0.30(34)	2.40	1.05
DOX-PC-DHI-UVA	0.09 ± 0.01(60)	0.59 ± 0.02(29)	2.36 ± 0.11(11)	0.47	1.02
DOX	0.13 ± 0.02(40)	1.06 ± 0.05(25)	5.29 ± 0.26(35)	2.3	1.03
DOX-UVA	0.14 ± 0.01 (45)	1.00± 0.05(32)	4.26 ± 0.21(23)	1.37	0.99

For various systems, the standard deviation (SD) of time-resolved decay measurements is shown. The amplitudes corresponding to the relevant decay components are shown within the parentheses.

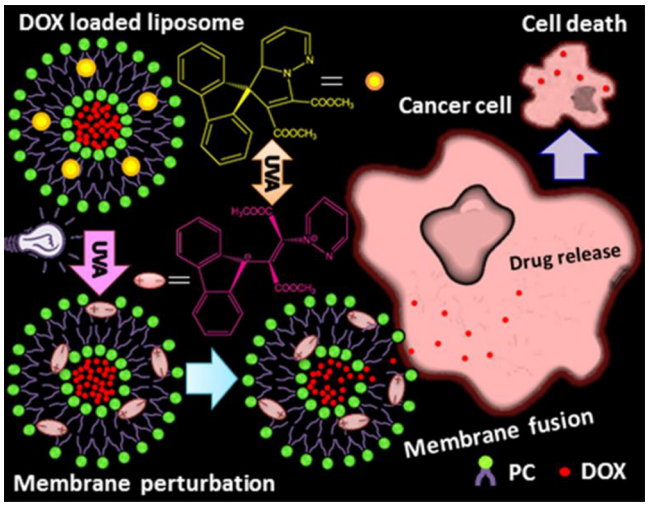
higher inter-liposome distance. Fig. 4(b) and (c) gave the morphological changes of liposomes due to UVA irradiation. Fig. 4(b) shows the spherical shape of liposomes in dark which confirmed that encapsulation of DHI doesn't hampered the morphology of liposome. When irradiated with UVA light, the liposomes of larger sizes and distorted shapes had been observed after photo isomerization. In a word, their liposome structures were still intact and exhibited no solubilized membrane even after being irradiated by UVA light. This finding is consistent with the results obtained by our time resolved spectroscopic studies.

3.3. Photo-triggered release and photoinduced cytotoxicity assay

Based on the photoresponsive properties and microstructural change investigated above, DHI-liposome could be considered as photoresponsive drug delivery system. As the liposome were closed and contained an inner aqueous compartment hence they showed the ability to entrap water-soluble dyes. The hydrophilic cargo drug DOX was used as a model drug, which was loaded into the internal aqueous phase of the liposome. Fig. 5(a) shows the release behavior of DOX from PC-DHI by UVA irradiation. The amount of DOX released out from the PC-DHI into the external medium was proportional to the change in fluorescence intensity. A burst release is found to occurred upon UVA irradiation at the 30 min followed by a slower sustained release up to 1 h, which is comparable to the other drug delivery systems [43,54]. We have found the spontaneous release of DOX was observed in the group without UVA irradiation as DOX absorbed on the surface of the liposome diffused to the outside medium when the samples were dispersed in aqueous solution. It has to be noted that, the leakage of DOX in the group without UVA irradiation was not entirely attributed to the burst release. The anticancer activity of the released DOX was further tested in vitro against HeLa cells. Although HeLa cell is quite older but still drug release from vehicles has been executed on HeLa as model cancer cell [55,56]. As our judgement is along the line of recent literature hence we have considered HeLa as model cancer cell. The data showed that (Fig. S4) released from the PC-DHI complex was capable of killing HeLa cells which implied that the encapsulation does not cause any major changes in the activity of the drug. Highest cell death was observed in the case of the release obtained from UVA-exposed PC-DHI-DOX system. This data correspond to the in vitro drug release study (Fig. 5(a)) where maximum amount of drug was released in the case of UVA-exposed PC-DHI-DOX system. Safety and efficacy are the necessary features that to be taken care of when investigating the potential use of the PC-DHI liposome as a drug delivery system. From the decades, liposomes are used for the drug delivery system because of its low toxicity, however photochromic agents used for triggering delivery vehicles are not always found to be biocompatible [57]. Hence, the cytocompatibility of the carrier PC-DHI was first evaluated against human epidermal keratinocyte cells (HaCaT cell line). The power of the UVA source and time of UVA exposure was also optimized using the same cell line (data not shown). For the cytocompatibility study, the PC and PC-DHI samples were directly added to the cells and the viability of the cells was measured by MTT (Fig. S5). From the data it was evident that PC-DHI has no detrimental effect on the viability of the HaCaT cells. The therapeutic efficacy of the drug loaded liposome was evaluated against cervical cancer cell line HeLa by exposing the cells directly to the PC-DHI-DOX in presence or absence of UVA (Fig. 5(b)). In this case also, the cell viability for PC-DHI was reduced only by 6 percentages than that of control indicating the cytocompatibility of PC-DHI formulation. It was observed that the cytotoxicity of DOX loaded-liposome (PC-DHI-DOX) was markedly enhanced following UVA irradiation in HeLa cells. Cell viabilities were about 60% after 24 h incubation with PC-DHI-DOX without UVA irradiation. In comparison, they decreased by more than 40% with UVA irradiation. UVA light induced a low cytotoxicity, only 5% compared to PC-DOX in dark, indicating DHI is solely responsible for higher delivery of the drug to the cell leading to more cytotoxicity upon UVA exposure.

3.4. Photoinduced cellular uptake

Further, the DHI-assisted delivery of the drug to the cells under UVA exposure was confirmed both qualitatively as well as quantitatively by the help of confocal microscopy and flow cytometry. Qualitative analysis of the confocal fluorescence micrographs clearly showed that under UVA exposure, cellular uptake of DOX in the HeLa cells treated with PC-DHI-DOX was quite higher with respect to the other three systems as evident from the variation in intensity of red colour in Fig. 5(c). The flow cytometry based quantitative analysis (Fig. 5(d)) revealed that% of cell populations that underwent DOX uptake was higher (98.3%) in the case cells treated with PC-DHI-DOX under UVA exposure in comparison to PC-DHI-DOX treated cells in dark condition (81.8%). Cells treated with PC-DOX shows no significant changes upon UVA irradiation. These findings altogether indicates higher DOX delivery by UVA trigger PC-DHI liposome (Scheme 2). The results presented in this study indicated that DHI encapsulated liposome could serve as a safe and promising drug delivery vehicle.



Scheme 2. Schematic representation of the photo-triggered release of DOX payload from PC liposome for controlled drug delivery.

4. Conclusions

In this study, we have investigated the photo-controlled alteration of liposome (L-α-phosphatidylcholin) dynamics and morphology via the incorporation of a new class of synthesized photochromic material, dihydroindolizine (DHI). The light-induced reversible pyrroline ring opening (zwitterion form) and the thermal back recovery reaction are responsible for its photochromism. We have demonstrated that structural conversion of DHI from closed to open isomer can fluctuate or defect the liposomal membrane by mechanical stress and hence responsible for fabrication of light trigger drug delivery systems. This destabilization causes an increase in the membrane permeability without complete disruption or solubilization of the liposome under irradiated conditions was manifested by time-resolved fluorescence spectroscopy of ANS bounded to liposome. A faster solvation dynamics in liposome upon light exposure compared to the dark condition rules out the presence of membrane destruction which has also been evidenced by time resolved polarization gated spectroscopy studies. Absence of an isoemissive point in TRANES further rules out the heterogeneity in the residence of ANS molecules upon destabilization of bilayer. The fusion between the liposomes leading to morphological changes under UVA irradiation was confirmed by using SEM as well as by FRET technique by incorporating ANS and DOX in different sets of liposome. The ability of DHI toward its photoresponsive properties and subsequently leading to microstructural change fabricates it to be considered for potential photoresponsive drug delivery system. Furthermore, the liposome had a strong and quick interaction with HeLa cells and enhanced significant cytotoxicity to the cells upon UVA irradiation. Confocal fluorescence microscopic and flow cytometry studies also revealed that the light triggered DHI encapsulated liposome have high drug delivery efficiency into HeLa cells. Overall, our study showed that these DHI encapsulated liposomes have potential application as a smart photosensitive drug delivery system for cervical carcinoma.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.colsurfb.2017.11.035.

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